Cell Stem Cell
Article

EGFR/Ras/MAPK Signaling Mediates Adult Midgut Epithelial Homeostasis and Regeneration in *Drosophila*

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DOI 10.1016/j.stem.2010.11.026

SUMMARY

Many tissues in higher animals undergo dynamic homeostatic growth, wherein damaged or aged cells are replaced by the progeny of resident stem cells. To maintain homeostasis, stem cells must respond to tissue needs. Here we show that in response to damage or stress in the intestinal (midgut) epithelium of adult *Drosophila*, multiple EGFR ligands and *rhomboids* (intramembrane proteases that activate some EGFR ligands) are induced, leading to the activation of EGFR signaling in intestinal stem cells (ISCs). Activation of EGFR signaling promotes ISC division and midgut epithelium regeneration, thereby maintaining tissue homeostasis. ISCs defective in EGFR signaling cannot grow or divide, are poorly maintained, and cannot support midgut epithelium regeneration after enteric infection by the bacterium *Pseudomonas entomophila*. Furthermore, ISC proliferation induced by Jak/Stat signaling is dependent upon EGFR signaling. Thus the EGFR/Ras/MAPK signaling pathway plays central, essential roles in ISC maintenance and the feedback system that mediates intestinal homeostasis.

INTRODUCTION

Homeostasis and regeneration in adult tissue has long fascinated biologists and clinicians alike. The discovery of resident somatic stem cells identified the source of the remarkable regenerating ability in some of adult human tissues, such as blood, skin, hair, and the digestive tract (Fuchs, 2009). However, how stem cells respond to tissue needs remains poorly understood (Pellettieri and Sánchez Alvarado, 2007). In particular, how stem cells are activated (for growth, proliferation, and differentiation) to regenerate new tissues after tissue injury, stress, or normal wear and tear is still unclear in most cases.

Homeostasis in the human small intestine and colon is mediated by intestinal stem cells (ISCs) that reside in the crypts of Lieberkühn (Barker et al., 2007; Radtke and Clevers, 2005). ISCs proliferate and differentiate to give rise to new functional epithelial cells in order to replenish cell loss from the villi. This dynamic process is intimately linked to the development of colorectal carcinoma (CRC), the second leading cause of cancer mortality in the western world (Radtke and Clevers, 2005). Oncological studies have established a genetic model for CRC development involving multiple steps: mutations in the *Adenomatous polyposis coli* (*Apc*) gene result in the activation of WNT signaling, which promotes the formation of small adenomas in the form of polyps. Subsequent mutations in KRAS, BRAF, p53, MLH1, or TGF-β signaling promote the formation of carcinomas, and finally additional mutations drive tumor metastasis (Vogelstein et al., 1988; Walther et al., 2009). Activation of receptor tyrosine kinases, particularly the epidermal growth factor receptor (EGFR), is believed to be an early event in the development of colon adenomas. Ectopic activation of EGFR signaling can cause intestinal and colonic hyperplasia, a likely precursor to ademona formation (Calcagno et al., 2008; Sandgren et al., 1990). Consistently, genetic studies have shown that ectopic activation of the EGFR pathway can accelerate tumor progression in the $Apc^{Min/+}$ genetic background (Bilger et al., 2008; Haigis et al., 2008; Phelps et al., 2009). Activating mutations in KRAS (codon 12, 13, or 61, which permanently lock it into the GTP-bound state) and BRAF ($BRAF^{V600E}$) are among the most common mutations found in colon cancer samples (Andreyev et al., 1998; Fransén et al., 2004; Roth et al., 2010). Furthermore, partial loss of function of EGFR ($Egfr^{wa2}$) severely impaired adenoma formation in $Apc^{min/+}$ mice (Roberts et al., 2002). Monoclonal antibodies against EGFR (*panitumumab* or *cetuximab*) are effective in treating CRC, provided that activating mutations in downstream KRAS or BRAF are not present, further emphasizing the critical role for EGFR signaling during CRC development (Amado et al., 2008; Di Nicolantonio et al., 2008). Developmentally, neonatal mice lacking EGFR function develop disorganized crypts in the gastrointestinal tract (Threadgill et al., 1995). Despite these many indications of its importance, the precise functions of EGFR signaling in normal gut homeostasis in mammals are poorly understood, making studies in model systems like *Drosophila* potentially informative.

As in the human intestine, the *Drosophila* adult midgut epithelium also undergoes rapid turnover, a dynamic process mediated by thousands of intestinal stem cells (ISCs) (Micchelli

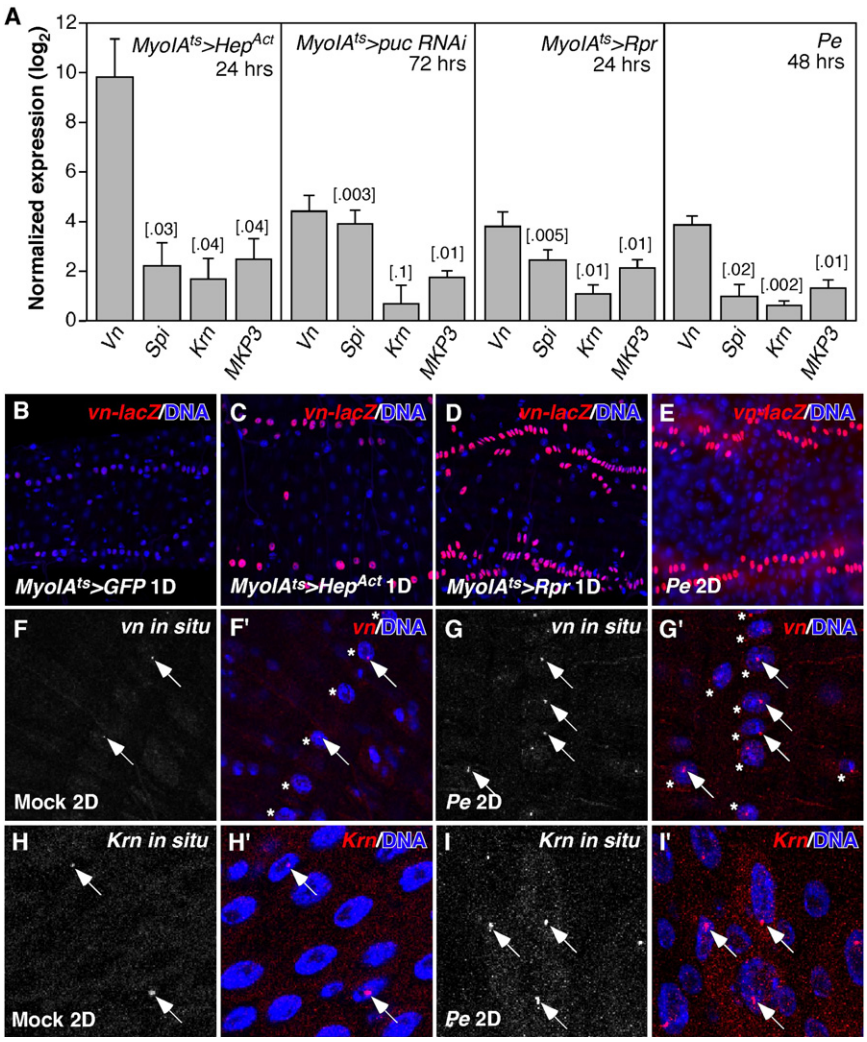


Figure 1. *Drosophila* EGFR Ligands Are Induced in the Regenerating Adult Midgut

(A) RT-qPCR quantification of *Drosophila* EGFR ligands (*vn*, *spi*, and *Krn*) and *MKP3* (MAP kinase phosphatase-3) mRNA expression in the regenerating midgut. The midgut was induced to regenerate by activating the JNK pathway in the ECs (*MyoIA*^{ts} > *Hep*^{Act}, 24 hr or *puc RNAi*, 72 hr) or inducing EC apoptosis (*MyoIA*^{ts} > *Rpr,* 24 hr) or *Pe* infection (48 hr). Error bars indicate standard deviation (STDEV) and p values (t test) are shown in brackets.

(B–E) Expression of *vn-lacZ* reporter in control (B) or regenerating posterior midguts (C–E). Two of the four rows of circular visceral muscle cells (VM) were shown.

(F and G) *vn* fluorescent in situ hybridization. The strongest *vn* signals were in the nucleus (arrows) of VMs (asterisks), most probably the loci of *Vn* transcription.

(H and I) *Krn* fluorescent in situ hybridization. The strongest *Krn* signals were in the nucleus of ECs (arrows).

In mock-infected control midguts, *vn* and *Krn* were expressed at low levels in the VM and ECs, respectively (F, H).

and Perrimon, 2006; Ohlstein and Spradling, 2006). In the fly midgut epithelium, basally localized intestinal stem cells divide, renew themselves, and give rise to progenitors called enteroblasts (EBs). In contrast to transit amplifying cells in mammalian intestinal crypts, *Drosophila* EBs appear not to proliferate, but directly differentiate into two conserved cell types, the absorptive enterocytes (ECs) and the secretory enteroendocrine cells (EE). Genetic studies show that the *Drosophila* Notch and WNT pathways play conserved roles in the self-renewal and proliferation of ISCs (Bardin et al., 2010; Lee et al., 2009; Lin et al., 2008; Ohlstein and Spradling, 2007). With this simple model, we and others previously demonstrated a feedback regulatory mechanism for maintaining adult tissue homeostasis. In this case, cell loss, damage, or stress in the midgut epithelium triggers the expression of *Unpaired* (*Upd*) cytokines by differentiated enterocytes, and these signals activate Jak/Stat signaling in intestinal stem cells to promote their proliferation and differentiation (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Cronin et al., 2009; Jiang et al., 2009). This feedback provides a truly homeostatic mechanism for tissue maintenance in the *Drosophila* midgut and may explain in general how stem cells respond to tissue needs in other organs and organisms.

In the present study we demonstrate that, in response to gut epithelial damage or stress in *Drosophila*, multiple EGFR ligands and several *rhomboids* are induced, and these activate the EGFR/RAS/MAPK pathway in ISCs. In parallel with Upd/Jak/Stat signaling, the activation of EGFR signaling promotes the proliferation of ISCs and their subsequent differentiation into mature midgut enterocytes, thus promoting gut self-renewal.

RESULTS

Damage or Infection of the Midgut Induces EGFR Signaling

To test whether EGFR signaling is induced in the regenerating *Drosophila* adult midgut, we assayed the expression of EGFR ligands in whole midguts via RT-qPCR. We induced midgut epithelium regeneration by expressing the cell death gene reaper (*Rpr*), or activated JNKK (*Drosophila Hep*^{Act}), or RNAi against *puckered* (*puc;* a feedback inhibitor of JNK signaling) in the enterocytes by means of the EC-specific-inducible *Gal4* driver, *MyoIA*^{ts}. Alternatively, we fed flies a pathogenic bacteria, *Pseudomonas entomophila* (*Pe*). As we showed previously, EC apoptosis, JNK activation, and enteric *Pe* infection all induce compensatory ISC proliferation and midgut epithelial regeneration (Jiang et al., 2009). We found that three *Drosophila* EGFR ligands, *vein* (*vn*), *spitz* (*spi*), and *Keren* (*Krn*), were induced in these regenerating midguts (Figure 1A). Regenerating midguts

also induced the expression of MAP Kinase Phosphatase 3 (MKP3), a downstream target of *Drosophila* EGFR signaling (Figure 1A). We examined the expression pattern of *vn* by using the *vn-lacZ* reporter. Weak expression was observed exclusively in the visceral muscle cells (VM) of control midguts, similar to its expression in the larval midgut (Figure 1B; Jiang and Edgar, 2009). *vn-lacZ* expression was highly induced in the VM of the regenerating midgut (Figures 1C–1E). The induction of *vn* expression in response to *Pe* infection was further confirmed by *vn* fluorescent in situ hybridization (Figures 1F and 1G). The strongest signals were found in the nuclei of circular and longitudinal visceral muscle cells, appearing as intense foci, probably the loci of *vn* transcription (Figures 1F and 1G). Similarly, the activation of apoptosis and JNK signaling in the ECs also induced *vn* expression in the VM (data not shown). However, in the case of ectopic JNK activation ($MyoIA^{ts}$ > Hep^{Act}), strong *vn* induction was also observed in the ECs (Figures S1A and S1B available online), where strong signals were also found in the cytosol. Induction of *vn* in the ECs by Hep^{Act} is consistent with the much higher *vn* induction in these midguts detected by RT-qPCR (Figure 1A). Fluorescent in situ hybridization further revealed that *Krn* was induced in the ECs in response to *Pe* infection (Figures 1H and 1I). The strongest signal appeared as intense foci in EC nuclei. In contrast, a reporter for *spi* ($spi\text{-}Gal4^{NP0261}$) was mainly expressed in small progenitor cells, with low levels of expression also observed in some ECs (Figures S1C and S1C′).

Drosophila rhomboids encode intramembrane proteases that cleave and activate some EGFR ligands, including *Spi* and *Krn* (Urban et al., 2002). We quantified the expression of all seven *rhomboid*-like genes in the midgut by RT-qPCR and observed modest upregulation of *rho*, *rho2*, *4*, and *6* in regenerating midguts (Figure S2A). We also examined the expression of *rho* with the rho^{X81}*-lacZ* reporter. *rho-lacZ* was weakly expressed in the VM (data not shown) but not in the epithelial cells of controls (Figure S2B). Although *rho-lacZ* expression in the VM did not change after infection (data not shown), its expression was induced in the ECs (Figures S2C–S2E). The induction of *rho* in the ECs in response to *Pe* infection was confirmed by in situ hybridization (Figures S2F and S2G).

The induction of multiple EGFR ligands and *rhos* in the midgut was also detected when flies were infected with another pathogenic bacteria, *ECC15* (Buchon et al., 2009b). We reasoned that the induction of these factors probably activates EGFR signaling. To test this, we examined the activity of mitogen-activated protein kinase (MAPK), a downstream effector of EGFR, by using antibodies against the diphosphorylated, active form of MAPK, termed dpERK (Gabay et al., 1997). Staining for dpERK in control midguts revealed that MAPK was mainly active in ISCs but was weak or absent in the EBs (Figure 2A; Figures S3A–S3A″). Brief *Pe* infection (1 day) led to increased dpERK in both ISCs and EBs (Figures 2B and 2B′), suggesting that *Pe* infection induced the activation of MAPK in midgut progenitor cells. Interestingly, MAPK activity in the progenitor cells decreased after 2 days of *Pe* infection, and ectopic MAPK activity was observed in newly formed pre-ECs (Figures 2C and 2C′). This downregulation in progenitors is probably the result of increased expression of MKP3, a negative regulator of MAPK (Figure 1A; Rintelen et al., 2003). Consistent with the activation of MAPK in midgut progenitors, ectopic induction of strong EGFR ligands ($MyoIA^{ts}$ > *sSpi*) activated MAPK only in the progenitor cells, but not in the mature ECs (Figures 2D and 2D′). However, activated Ras ($esg^{ts}F/O$ > Ras^{V12}) led to strong cell-autonomous activation of MAPK in both progenitors and large polyploid ECs (Figures 2E and 2E′). This suggests that differentiated ECs lack a critical component of the EGFR pathway upstream of Ras and are therefore unable to respond to EGFR ligands. One possibility is that ECs downregulate EGFR as they differentiate.

EGFR Activates ISCs through RAS/RAF/MAPK Signaling

We previously reported that EGFR signaling drives the proliferation of adult midgut progenitors (AMPs) in the larval gut and showed that VM-derived *Vn* is required for AMP proliferation during early larval development (Jiang and Edgar, 2009). By using an inducible visceral muscle driver, $24B^{ts}$, we overexpressed *Vn* specifically in adult VM and observed a mild increase of mitotic ISCs (Figure 3A). Thus VM-derived *Vn* is sufficient to induce ISC proliferation. The mild effect on ISC proliferation is probably because *Vn* is a weak EGFR ligand (Schnepp et al., 1998). Next, we ectopically activated EGFR signaling in the ISCs by expressing the strong EGFR ligands, *sSpi* or *sKrn* (Reich and Shilo, 2002; Schweitzer et al., 1995), activated Egfr (*λTOP*) (Queenan et al., 1997), or activated Ras (Ras^{V12}) (Karim and Rubin, 1998) by using a lineage induction system, $esg^{ts}F/O$. In the $esg^{ts}F/O$ system, progenitor cells and all of their newborn progeny express *Gal4* and *UAS*-linked *Gal4* targets, including the *UAS-GFP* marker (Jiang et al., 2009). We then examined their effects on ISC proliferation. Activation of EGFR signaling induced increased ISC division (Figure 3B), resulting in the generation of many new midgut cells, including EC-like GFP^+ cells (Figures 3D–3F). Most of these large GFP^+ cells were positive for PDM-1, a marker for fully differentiated ECs (Figures 3F–3F″). Therefore, EGFR/Ras signaling does not suppress EC differentiation. In addition, we found that knocking down *Cbl*, a negative regulator of EGFR signaling (Hime et al., 1997; Meisner et al., 1997), by *Cbl RNAi* ($esg^{ts}F/O$ > *Cbl RNAi*), also induced ISC proliferation (Figure 3B; Figure S4B). Prolonged activation of EGFR signaling resulted in severely hyperplasic midguts (Figure S8D).

We also induced EGFR ligands in mature ECs ($MyoIA^{ts}$ > *sSpi* or *sKrn*). This treatment similarly promoted ISC proliferation, demonstrating that paracrine EGF signaling is able to activate ISC division (Figure 3B). In fact, the source of ectopic EGFR ligands did not seem to be important. No matter where *Vn*, *sSpi*, or *sKrn* were induced (VMs, ECs, or progenitors), they were always capable of inducing dramatic ISC proliferation (data not shown).

To ask which downstream effectors of EGFR are responsible for inducing ISC proliferation, we ectopically expressed pathway-specific *Ras* variants (Ras^{V12S35} or Ras^{V12G37}) in midgut progenitor cells (Karim and Rubin, 1998). Ras^{V12S35}, which specifically activates the MAPK pathway, was able to promote ISC proliferation, whereas induction of Ras^{V12G37}, which preferentially activates the PI3K/AKT pathway, had no effect on ISC proliferation (Figure 3B). Activated Raf (Raf^{gof}) also promoted ISC proliferation (Figure 3B), and coexpressing *MKP3* largely inhibited ectopic ISC proliferation induced by Ras^{V12} (Figure 3B). Furthermore, depleting *Capicua* (*Cic*) ($esg^{ts}F/O$ > *Cic RNAi*),

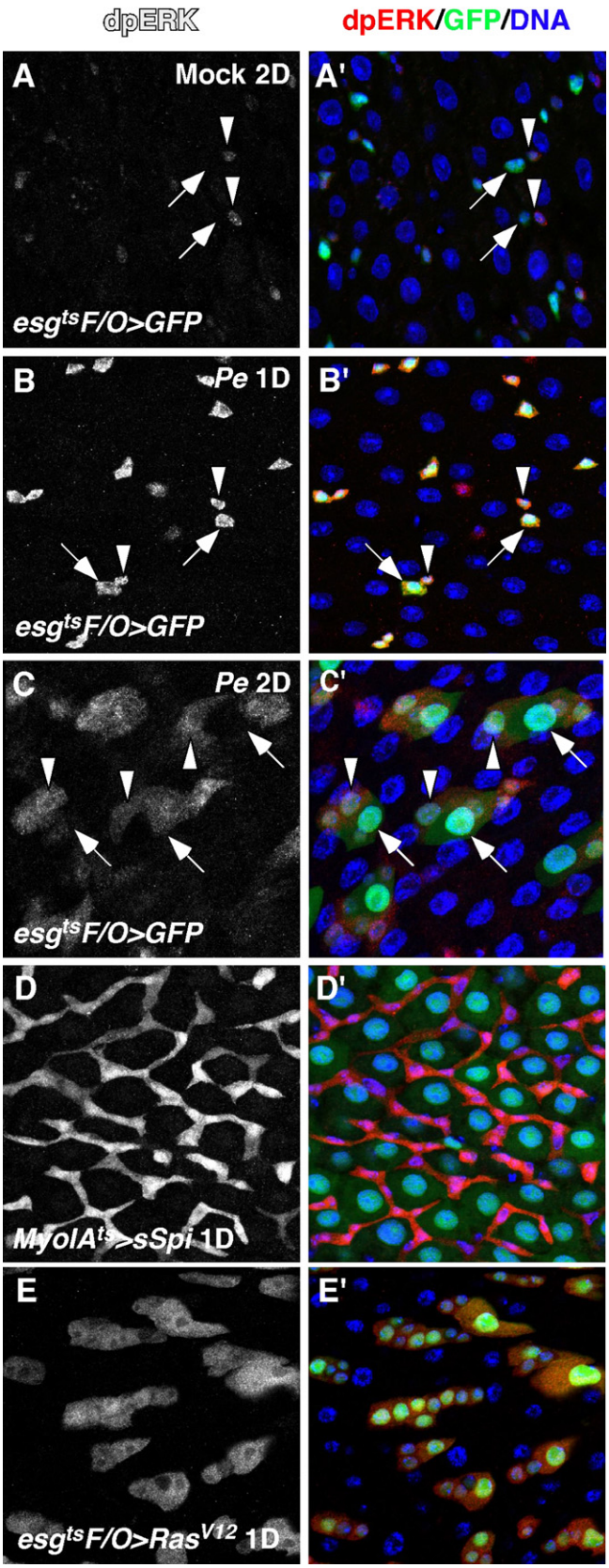


Figure 2. MAPK Is Activated in the Regenerating Midgut

The activity of *Drosophila* MAPK was assayed by anti-dpERK staining.

(A and B) MAPK activity in the mock-infected control midgut (A). MAPK activity after infecting with *Pe* for 1 day (B). ISCs and EBs were marked by *esgGal4*-driven GFP expression and indicated by arrowheads and arrows, respectively (A, B).

(C) MAPK activity after infecting with *Pe* for 2 days. Differentiating ECs (pre-ECs, medium nucleus) and newly formed mature ECs (large nucleus) were indicated by arrowheads and arrows, respectively.

(D) MAPK activation induced by ectopic expression of *sSpi* (*MyoIA^{ts} > sSpi*).

(E) Cell-autonomous MAPK activation induced by activated Ras (*esg^{ts}F/O > Ras^{V12}*).

a transcriptional repressor downstream of MAPK pathway (Astigarraga et al., 2007), also induced ISC proliferation (Figure 3B; Figure S4C). We conclude that EGFR signaling induces ISC proliferation specifically through Ras, Raf, and MAPK, rather than via PI3K or another effector pathway.

EGFR Signaling Is Required for ISC Proliferation and Midgut Regeneration

To further explore the role of EGFR signaling in the midgut, we generated mosaic ISC clones homozygous for *ras$^{\Delta c40b}$*, a null allele (Schnorr and Berg, 1996), or *Egfr* (*Egfr* null, *Egfr$^{[CO]}$*) (Clifford and Schüpbach, 1989), or both *ras* and *stat* function (*ras* and *Stat92E* double null mutants, *ras$^{\Delta c40b}$*, *stat^{397}*) (Silver and Montell, 2001) via the MARCM system (Lee and Luo, 2001). We then quantified the size of marked ISC clones at intervals after clone induction. Although the initial growth of *ras* and *Egfr* mutant ISC clones was normal, their long-term proliferation was severely compromised (Figures 4A–4E). For *ras* and *stat* double mutant, the clones were not only small, but also lacked ECs (Figure 4D), a phenotype consistent with Jak/Stat's critical role for ISC differentiation (Beebe et al., 2010; Jiang et al., 2009). Consistent with the EGFR pathway's essential role in ISC proliferation, midgut renewal after *Pe* infection was completely inhibited when EGFR signaling was suppressed in the progenitor cells by *Egfr RNAi* (Figures 4G–4J). Furthermore, prolonged EGFR suppression in healthy animals (4 weeks) led to almost complete loss of enteroblasts (esg^{+}, Su(H)$^{+}$) and ~33% reduction of intestinal stem cells (esg^{+}, Su(H)$^{-}$) (Figures 4F and 4I). In the short term, however, EGFR suppression did not significantly alter the number of ISCs, but probably only prevented their growth and division. Interestingly, old ECs generated before the induction of lineage marking were still present in these aged midguts (~1 month, Figure 4I), suggesting that EC loss were also partially inhibited.

Next we tested whether EGFR signaling is required for compensatory ISC proliferation and midgut epithelium regeneration induced by *Pe* infection. We first examined the growth of control ISC clones in *Pe*-infected midgut and observed large ISC clones (~7 cells/clone) 2 days after clone induction (Figure 4E). However, the ISC clones lacking *ras* or *Egfr* function were much smaller (~3 cells/clone). Like the long-term *ras* or *Egfr* mutant ISC clones in noninfected midguts, these clones did not grow even after the flies had recovered from *Pe* infection for about a week (Figure 4E). Quantification of midgut mitotic indices revealed that *Pe*-induced compensatory ISC proliferation was completely inhibited when *Egfr* or *Raf* was knocked down (*esg^{ts}F/O > Egfr RNAi* or *Raf RNAi*; Figure 4K). Furthermore, although *Pe* infection almost completely eliminated old

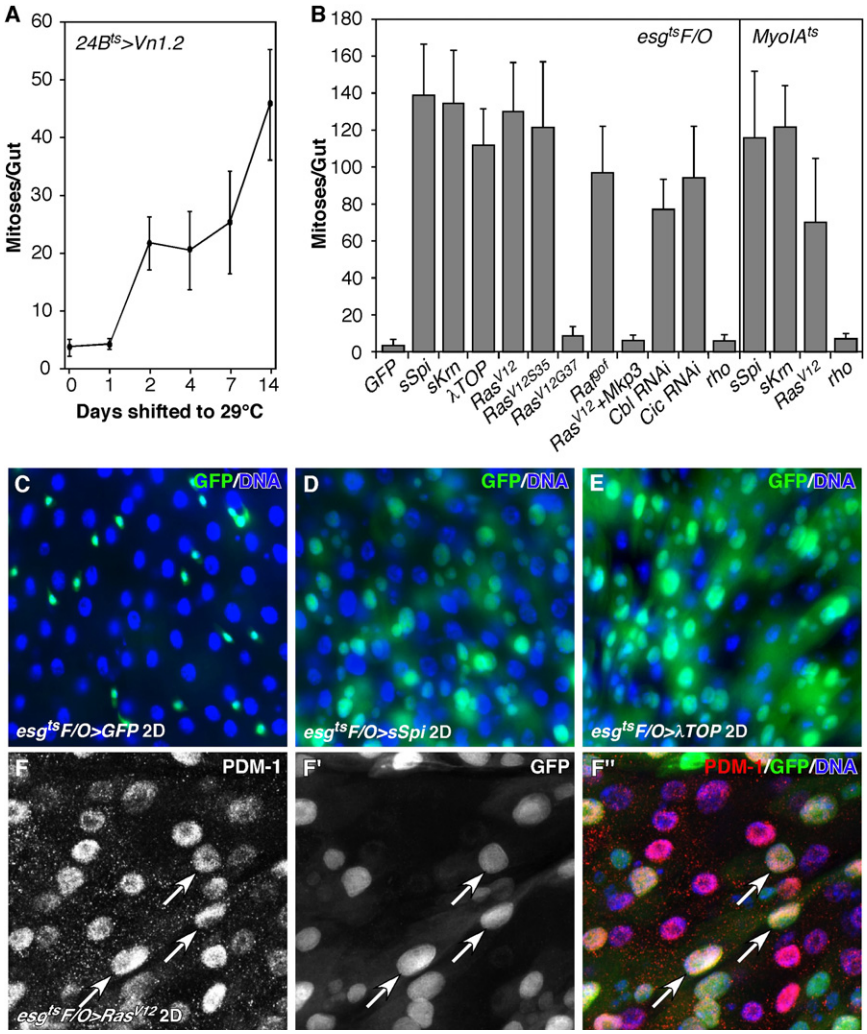


Figure 3. EGFR Signaling Promotes ISC Proliferation and Midgut Growth

(A) Ectopic ISC proliferation induced by *Vn*. *Vn* was induced in the midgut via the inducible VM-specific driver $24B^{ts}$.

(B) ISC proliferation induced by activated EGFR signaling. Transgenes were induced in the midgut for 2 days via the $esg^{ts}F/O$ or $MyoIA^{ts}$ system. Midguts were scored for $PH3^{+}$ mitotic figures in both (A) and (B). Error bars represent standard deviation (STDEV) in (A) and (B).

(C–E) Adult midgut growth measured via the $esg^{ts}F/O$ system. Both *sSpi* (D) and *λTOP* (E) promoted significant new midgut cell formation.

(F) Ras^{V12} also promoted the formation of new mature midgut cells. Most of the newly formed large polyploid midgut cells (GFP^{+}, arrows) were positive for mature EC marker, PDM-1.

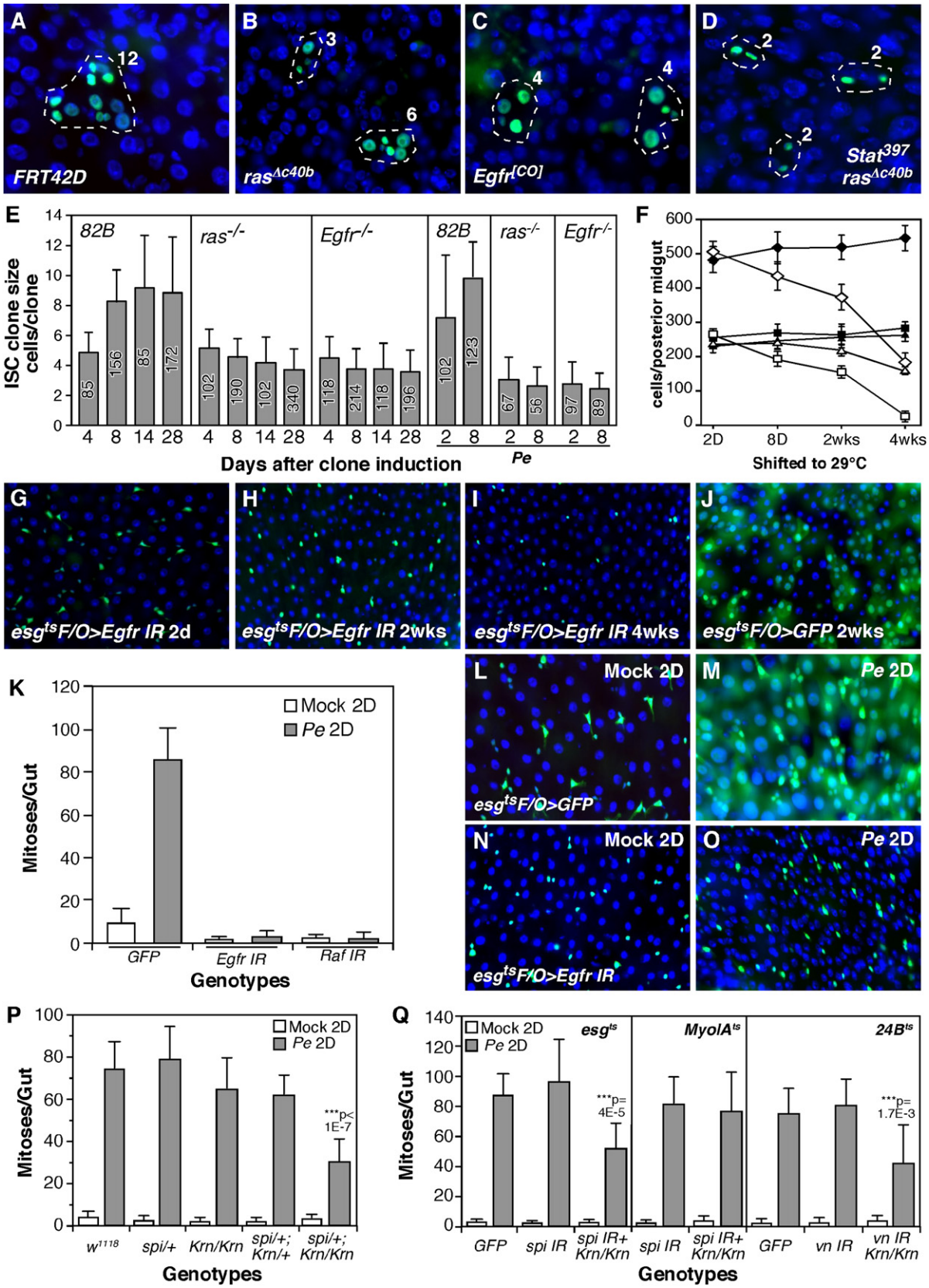
ECs and induced midgut epithelial regeneration in controls (Figures 4L and 4M), suppression of EGFR signaling largely inhibited midgut epithelium regeneration (Figures 4N and 4O; Figure S5). In both cases, however, large numbers of progenitor cells expressing these RNAis survived for the duration of the experiment. In summary, EGFR signaling is required for ISC proliferation during both normal midgut homeostasis and regeneration, such as that induced by *Pe* infection.

Multiple EGFR Ligands Function Redundantly to Activate ISC Proliferation

To examine the function of EGFR ligands and *rhomboid* during *Drosophila* midgut homeostasis and regeneration, we knocked down *spi*, *vn*, and *rho* individually in the midgut via RNAi and several midgut-specific drivers, including esg^{ts}, $MyoIA^{ts}$, and $24B^{ts}$. Inducing *spi RNAi* in midgut progenitors (esg^{ts} > *spi RNAi*), *vn RNAi* in visceral muscle cells ($24B^{ts}$ > *vn RNAi*), or *rho RNAi* in the ECs ($MyoIA^{ts}$ > *rho RNAi*) all significantly knocked down target gene expression (Figure S6A). In each case, however, these RNAi-depleted midguts appeared to be normal, even after long periods of gene knockdown (data not shown). We then orally infected the flies with *Pe* and quantified ISC proliferation. *Pe* infection-induced ISC proliferation also appeared normal in these RNAi-depleted midguts (Figure 4Q; Figure S6B). Finally we examined the regenerative response in the midguts of *Krn* ($krn^{27\text{-}7\text{-}B}$, viable null), *rho* ($rho^{A0544}$, viable partial loss-of-function), and *Star* (S^{d01624}, viable partial loss-of-function) mutants (Corl et al., 2009; McDonald et al., 2006). In these cases ISC proliferation induced by *Pe* infection was also normal (Figure 4P; Figure S6B).

In further tests we quantified *Pe*-induced ISC proliferation in *spi* and *Krn* double mutants. In this case we found that heterozygosity for *spi* in a *Krn* homozygous mutant background (spi^{A14}/+; $Krn^{27\text{-}7\text{-}B}/Krn^{27\text{-}7\text{-}B}$) significantly reduced *Pe*-induced ISC proliferation (Figure 4P). Our previous analysis indicated that this double mutant does not affect the development of the adult midgut progenitor (AMPs) in larvae (Jiang and Edgar, 2009), and quantification of esg^{+} cells indicated that these midguts had normal numbers of progenitor cells (data not shown). Hence, the suppression of ISC mitotic response suggests that *spi* and *Krn* function redundantly during midgut epithelium regeneration. To test which cell types are the source of *spi* expression, we knocked down *spi* expression with *RNAi*, driven either by the esg^{ts} driver (progenitor-specific) or the $MyoIA^{ts}$ driver (EC-specific) in a *Krn* mutant background. Knocking down *spi* in progenitor cells (esg^{ts} > *spi IR*, $Krn^{27\text{-}7\text{-}B}/Krn^{27\text{-}7\text{-}B}$) but not ECs ($MyoIA^{ts}$ > *spi IR*; $Krn^{27\text{-}7\text{-}B}/Krn^{27\text{-}7\text{-}B}$) significantly reduced midgut mitoses induced by *Pe* ingestion (Figure 4Q). We surmise that autocrine *spi* (from progenitor cells) and paracrine *Krn* (from ECs) function redundantly to promote ISC proliferation during midgut epithelium regeneration.

We next tested *vein* function, by using *RNAi* to deplete *vn* in the visceral muscle of *Krn* mutant animals, via the $24B^{ts}$ driver. Simultaneous loss of *Krn* and *vn* ($24B^{ts}$ > *vn IR*, $Krn^{27\text{-}7\text{-}B}/Krn^{27\text{-}7\text{-}B}$) significantly reduced the ISC proliferation (Figure 4Q),


A
12
FRT42D
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C
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Days after clone induction
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cells/posterior midgut
2D
8D
2wks
4wks
Shifted to 29°C
G
esgtsF/O>Egfr IR 2d
H
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esgtsF/O>GFP 2wks
K
Mock 2D
Pe 2D
Mitoses/Gut
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Egfr IR
Raf IR
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suggesting that *vn* and *Krn* also have overlapping function during midgut epithelium regeneration.

EGFR Signaling Is Required for ISC Proliferation Induced by Jak/Stat Signaling

Because both EGFR and Jak/Stat signaling are sufficient and required for midgut epithelium regeneration and both pathways are induced in the regenerating midgut (Figures 1–4; Buchon et al., 2009a; Cronin et al., 2009; Gabay et al., 1997; Jiang et al., 2009), we examined their epistatic relationship. We first ectopically activated EGFR signaling and examined the expression of the *Upd* cytokines by RT-qPCR. When activated EGFR ligand (*MyoIA^{ts} > sKrn*), activated Egfr (esg^{ts}*F/O > λTOP*), or activated Ras (esg^{ts}*F/O > Ras^{V12}*) were expressed in the midgut, all three Upd cytokines were induced, along with downstream target gene *Socs36E* (Figure 5A). Consistently, the *upd-lacZ* reporter was induced in the midgut epithelial cells by *Ras^{V12}* (Figures 5C and 5D). Similarly, when we ectopically activated EGFR signaling (*MyoIA^{ts} > Ras^{V12}*), the *upd3* reporter, *upd3.1-lacZ*, was induced in the ECs (Figures 5E and 5F). Accordingly, *Ras^{V12}* expression in the ECs was capable of inducing ISC proliferation (Figure 3B). The induction of cytokines and subsequent activation of Jak/Stat signaling probably depends on the levels of EGFR activation because the inductions by *sKrn* were much lower than that by activated EGFR (*λTOP*) or *Ras^{V12}* (Figure 5A). Moreover ectopic expression of *Vn (24B^{ts} > Vn)*, a weak EGFR ligand, did not induce cytokine expression (data not shown), though it did promote mild ISC proliferation (Figure 3A).

We next asked what signals might induce *Vn* expression in the visceral muscle. We observed increased nuclear STAT92E staining in the VM of *Pe*-infected midguts (Figures S7A and S7B), suggesting that Jak/Stat signaling was activated in the VM. Consistent with this, expression of the Jak/Stat reporter *10XSTAT-DGFP* increased dramatically in the VM after *Pe* infection (Figures S7C and S7D). Because the induction of *vn* coincided with enhanced cytokine signaling in the VM, we speculated that it might be the result of *Upds* (cytokine) released from the midgut epithelium. In testing this idea, we found that *vn* and the *vn-lacZ* reporter could be induced in the VM in response to EC-specific expression of *Upd* (*MyoIA^{ts} > Upd*) (Figures 5B, 5G, and 5H). Activating Jak/Stat signaling directly in the VM via the expression of *Drosophila Jak* (*24B^{ts} > Hop*) also induced comparable *vn* expression (Figure 5B). These experiments indicate that midgut epithelium-derived cytokines can activate Jak/Stat signaling and induce *vn* expression in the VM. However, we found that *Pe* infection could induce *vn* upregulation in the midguts of *Jak* mutants (*hop^{25}*, partial loss-of-function) or when *stat* was depleted in the VM (*24B^{ts} > Stat RNAi*; Figure S7E). These data indicate that, although activated Jak/Stat signaling can induce *vn*, Jak/Stat signaling is not required for *vn* induction in response to *Pe* infection.

Further epistasis tests showed that when EGFR signaling was activated in the background of reduced Jak/Stat signaling (esg^{ts}*F/O > sKrn + Stat* or *Dome RNAi*), its stimulatory effect on ISC proliferation was not diminished (Figure 6A; Figures S8D–S8F). Similar results were obtained when activated Egfr (*λTOP*) or Ras (*Ras^{V12}*) was coexpressed with *Stat* or *Dome RNAi* (data not shown). By using the MARCM technique, we induced activated Ras in ISCs mutant for Stat (+*Ras^{V12}*, *stat^{397}*) and analyzed their clonal growth. Loss of Jak/Stat signaling did not affect *Ras^{V12}*'s ability to drive the growth of large ISC clones (Figures 6F and 6G). However, in a similar experiment, clonal growth induced by the weak EGFR ligand, *Vn*, was largely inhibited by loss of *Stat* (Figures 6C, 6D, and 6K). These data suggest that the requirement of Jak/Stat signaling for ISC proliferation probably depends on the levels of EGFR activation, such that high-level EGFR activation is able to induce ISC proliferation independent of Jak/Stat signaling, whereas ISC proliferation induced by low-level EGFR activation (such as that induced by *Vn*) is largely dependent on Jak/Stat signaling.

In further experiments we found that ISC proliferation induced by ectopic *Upd* was completely inhibited when EGFR signaling was downregulated in the ISCs (Figure 6A). Knocking down *Egfr* or *Ras* completely inhibited the midgut hyperplasia phenotype that results from ectopic *Upd* expression (esg^{ts}*F/O > Upd + Egfr* or *Ras RNAi*; Figures S8G–S8I). Similar results were obtained in a clonal setting, with the *ras^{Δc40b}* mutant allele (Figures 6I–6K). Thus EGFR signaling is required for ISC proliferation induced by Jak/Stat signaling. However, activating Jak/Stat and EGFR signaling simultaneously induced a much higher ISC mitotic index than that induced by the activation of either pathway alone (*MyoIA^{ts} > Upd + sSpi*; Figure 6A), indicating that the two pathways can function synergistically to induce ISC proliferation. Like the Jak/Stat signaling (Beebe et al., 2010), EGFR signaling can also induce much higher rate of ISC proliferation when Notch signaling is inhibited

Figure 4. *Drosophila* EGFR Signaling Is Required for Midgut Homeostasis and Regeneration

(A–D) MARCM analysis of ISC clones. Wild-type (A) and mutant ISC clones (B–D) were induced with the MARCM system and examined 8 days later. The number of cells in each clone were indicated.

(E) Quantification of ISC clone sizes. The number of clones counted for each genotype were indicated inside each bar.

(F) Quantification of progenitor cells in the posterior midguts of GFP and EGFR knockdown. Progenitor cells (esg^{+}) were indicated by diamonds, EBs (esg^{+}, Su(H)$^{+}$) were indicated by squares, and presumed ISCs (esg^{+}, Su(H)$^{-}$) were indicated by triangles. Filled symbols, *esg^{ts} > GFP*; open symbols, *esg^{ts} > EGFR RNAi*.

(G–J) Midgut epithelium turnover assay. EGFR suppression inhibited midgut turnover (H, esg^{ts}*F/O > Egfr RNAi*). Furthermore, GFP^{+} progenitor cells were depleted after long-term EGFR knockdown (I). In control midgut, GFP were present in both progenitors and large polyploid cells (probably ECs) after 2 weeks (J, esg^{ts}*F/O > GFP*).

(K) Quantification of compensatory ISC proliferation induced by *Pe* infection. EGFR signaling was suppressed in the progenitor cells by esg^{ts}*F/O*-driven *Egfr* or *Raf RNAi*.

(L–O) Midgut turnover in mock (L, N) or *Pe*-infected (M, O) animals. Midgut turnover was assayed via the esg^{ts}*F/O* system.

(P and Q) Quantification of compensatory ISC proliferation in *spi*, *vn*, and *Krn* mutants. We used viable *Krn* null mutant (*Krn$^{27\text{-}7\text{-}B}$*), lethal *spi* null mutant (*spi^{A14}*, in a heterozygous background), *spi RNAi* knockdown in progenitors (*esg^{ts} > spi IR*) or ECs (*MyoIA^{ts} > spi IR*), or *vn RNAi* knockdown in VMs (*24B^{ts} > vn IR*). IR, inverted repeats.

Error bars represent STDEV in (E), (F), (K), (P), and (Q).

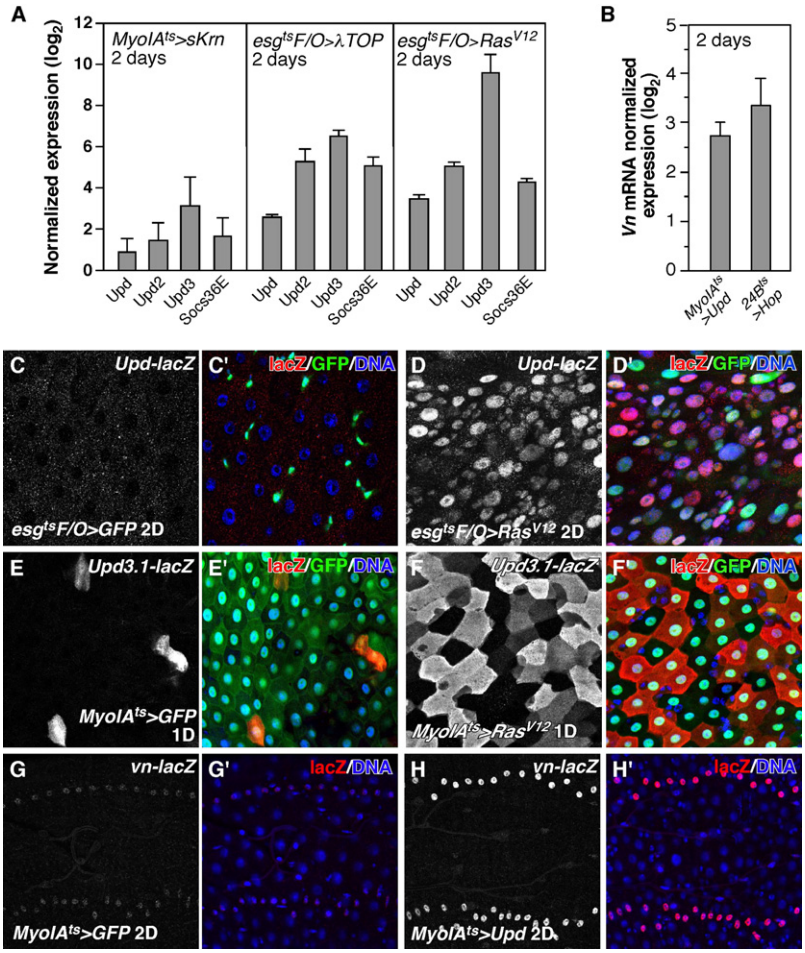

Figure 5. Induction of EGFR and Jak/Stat Signaling in the Midgut

(A) Activating EGFR signaling induced Jak/Stat signaling in the midgut. The expression levels of *Drosophila* cytokines (*upds*) and downstream target gene, *Socs36E,* in the midgut were analyzed by RT-qPCR.

(B) Induction of *vn* expression in the midgut by Jak/Stat signaling as quantified by RT-qPCR. Jak/Stat signaling was activated in the VM by ectopic expression of *Upd* in the ECs (*MyoIA^{ts}* > *Upd*) or *Hop* directly in the VM (*24B^{ts}* > *Hop*).

Error bars represent STDEV in both (A) and (B).

(C and D) Induction of the *upd-lacZ* reporter in the midgut epithelium by activated Ras (*esg^{ts}F/O* > *Ras^{V12}*, D).

(E and F) Induction of the *Upd3.1-lacZ* reporter in ECs by activated Ras (*MyoIA^{ts}* > *Ras^{V12}*, F).

(G and H) Induction of the *vn-lacZ* reporter in the VM by ectopic expression of *Upd* (*MyoIA^{ts}* > *Upd*, H).

(*esg^{ts}F/O* > *sKrn* + *N IR*; Figure 6A). Because *Notch* suppression increases stem cell pools, this suggests that both pathways primarily regulate ISC division, rather than ISC numbers.

Finally, we examined whether the induction of Upd/Jak/Stat and EGFR signaling by *Pe* infection depended on each other. We inhibited *Pe*-induced midgut epithelium regeneration by knocking down *Egfr* (*esg^{ts}F/O* > *Egfr RNAi*) or *Stat* (*esg^{ts}F/O* > *Stat RNAi*) and examined the expression of *upds* and *Socs36E* or Egfr ligands and *rhos* by RT-qPCR. The induction of Jak/Stat and EGFR signaling by *Pe* was normal in both cases (Figure 6L), suggesting that these two signaling pathways can be induced independently of each other by midgut damage (Figure 7).

DISCUSSION

EGFR Signaling Is Essential for ISC Growth and Division

These studies show that the EGFR pathway provides an essential mitogenic signal for ISC proliferation during midgut homeostasis and regeneration (Figure 4). Furthermore, ISC proliferation induced by Jak/Stat signaling depends on functional EGFR signaling (Figures 6A and 6H–6K; Figure S8G–S6I). The critical role of EGFR signaling in the fly midgut is consistent with its role during mammalian gut homeostasis and colorectal cancer development. EGFR signaling is required for the development, maintenance, and tumorigenesis of mucosal epithelium in the mouse GI tract (Roberts et al., 2002; Threadgill et al., 1995; Troyer et al., 2001). Antibodies targeting EGFR have been shown to be effective in treating colorectal cancer provided there are no activating mutations in downstream signaling components, such as KRAS or BRAF (Amado et al., 2008; Di Nicolantonio et al., 2008).

Our data also demonstrate that EGFR signaling is induced in response to damage in the *Drosophila* midgut and functions to promote ISC proliferation during midgut epithelium regeneration (Figures 1–3). In this capacity it is a central and essential component of the feedback mechanism for adult tissue homeostasis that we described previously (Figure 7; Jiang et al., 2009). Like EGFR ligands in *Drosophila*, two mammalian EGFR ligands, *epiregulin* and *amphiregulin*, have been reported to be upregulated in the gut epithelium after damage (Lee et al., 2004; Nishimura et al., 2008). Their expression is also increased in neoplastic lesions in the colon, suggesting a possible role in colon cancer development (Nishimura et al., 2008).

One of our more unexpected findings was that, whereas differentiating immature cells (preECs) were often positive for MAPK activity, fully differentiated midgut cells such as ECs were not (Figures 2C and 2C′). A potential explanation for this is that mature ECs lose EGFR or a downstream effector and thereby become unresponsive to EGFR ligands. This is consistent with our data showing that MAPK could be activated only in progenitor cells (ICSs and EBs) even when activated EGFR ligands (such as *sSpi*) were ectopically expressed at high levels (Figures 2D and 2D′). A similar mechanism may confine the activity of Jak/Stat signaling to the midgut progenitor cells (Beebe et al., 2010; Buchon et al., 2009a; Jiang et al., 2009). In this case *Domeless*, the receptor for the *Upd* cytokines, is expressed in the midgut progenitor cells but not in their progeny (Jiang et al., 2009). Switching off receptor expression for cytokines or growth factors may be one way to ensure that mature differentiated cells do not respond to these

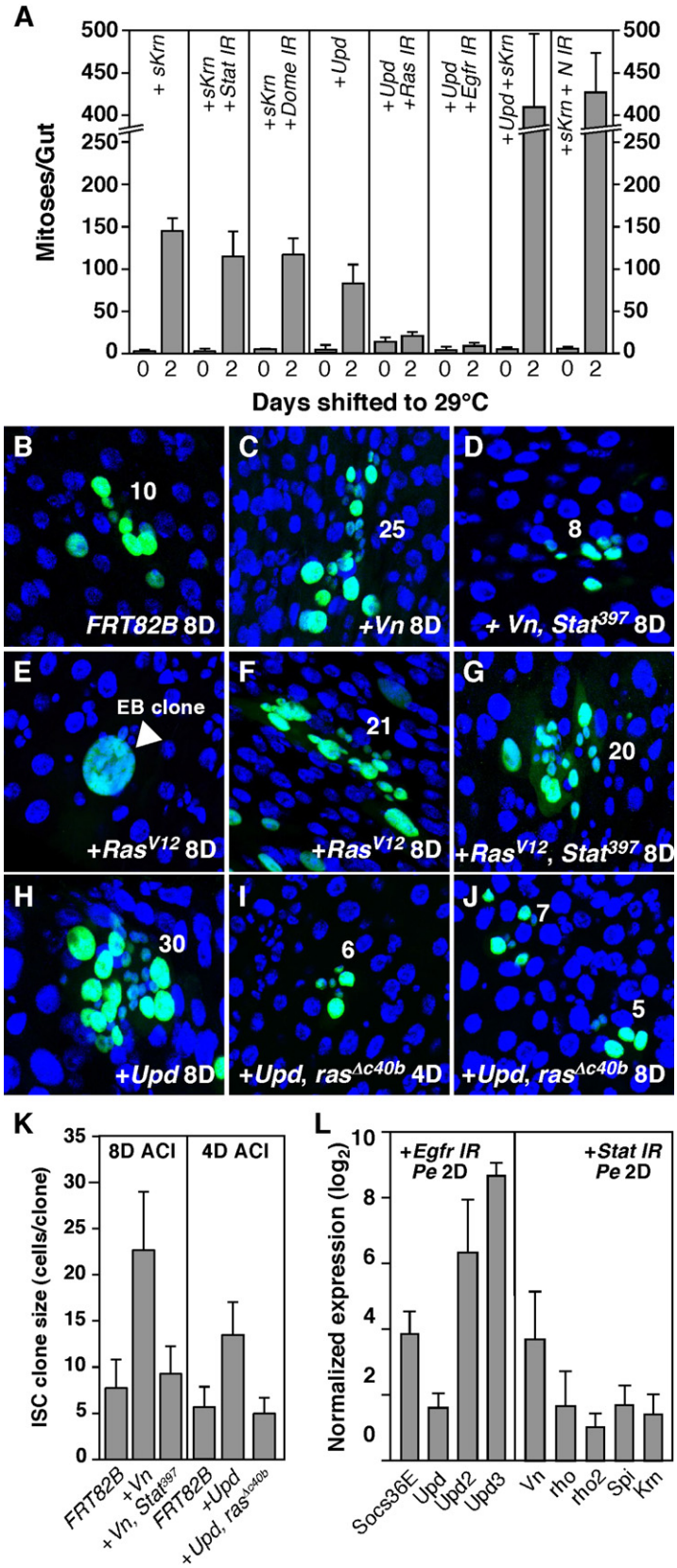

Figure 6. Jak/Stat-Induced ISC Proliferation Requires EGFR Signaling

(A) ISC proliferation induced by EGFR and Jak/Stat signaling. With the exception of coexpressing *sKrn* and *Upd* in the ECs (*MyoIA*^{ts} > *Upd* + *sKrn*), all the other ectopic expression experiments were performed with the *esg*^{ts}*F/O* driver. Midgut mitotic indices (PH3^{+}) were quantified after activating the transgenes for 2 days.

(B–J) ISC clonal assay. GFP-marked ISC clones were induced with the MARCM system and analyzed 4 or 8 days later. The sizes of the ISC clones were indicated. *Vn*-induced ISC proliferation is dependent on Jak/Stat signaling (B–D). Activated Ras (*Ras*^{V12})-induced ISC proliferation is independent of Jak/Stat signaling (F, G). Some EB clones overexpressing *Ras*^{V12} underwent extra round of endoreplication (E). *Upd*-induced ISC proliferation is dependent on EGFR signaling (H–J).

(K) Quantification of ISC clone sizes. The sizes of ISC clones were measured 4 or 8 days after clone induction (ACI) via the MARCM system.

(L) RT-qPCR analysis of the induction of Jak/Stat and EGFR signalings by *Pe* infection in the absence of either pathway (*esg*^{ts}*F/O* > *Stat* or *Egfr RNAi*).

Error bars represent STDEV in (A), (K), and (L).

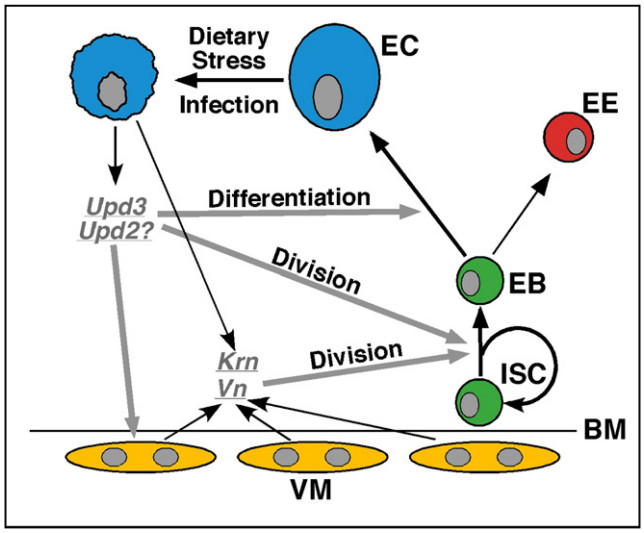

Figure 7. Updated Model for Midgut Homeostasis and Regeneration in *Drosophila*

Stressed or dying ECs induce the expression of fly cytokines (such as *Upd3* and *Upd2*) and EGFs (such as *Krn* and *Vn*) in the midgut, which activate the Jak/Stat and EGFR pathways in the midgut progenitor cells. Whereas EGFR signaling functions mainly to promote ISC proliferation, Jak/Stat signaling functions to promote both ISC proliferation and EB differentiation.

mitogenic cues. Despite this failsafe mechanism, the expression of Ras^{V12} was able to induce the cell-autonomous activation of MAPK (Figures 2E and 2E′) and the expression of *Upd3* in the ECs (Figures 5E and 5F), leading to a non-cell-autonomous stimulation of ISC proliferation (Figure 3B). This suggests that the downregulation of mitogen receptors upon differentiation may be important to throttle EGFR←→Jak/Stat positive feedback that might otherwise result in run-away signaling and ISC proliferation.

As with the Upd cytokines, we know little about how the *Drosophila* EGFR ligands are induced by stress or damage to the midgut epithelium. In the case of the Upds, potential activating stress signals span a very wide range, including induced apoptosis, autophagic cell death, JNK signaling, infection by pathogenic bacteria, colonization by nonpathogenic enteric bacteria, ingestion of detergents, oxidative stress inducers, DNA damaging agents, and even physical "pinching" of the epithelium (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Biteau et al., 2008; Buchon et al., 2009a; Cronin et al., 2009; Jiang et al., 2009). The signals capable of activating the EGFR ligands are likely to be just as diverse. Further genetic studies in the fly should be able to determine whether these stress responses are cell autonomous or a property of the epithelium as a tissue and to identify the genes and pathways involved. Given the critical roles of the mammalian Jak/Stat and EGFR pathways in regulating tissue homeostasis and cancer development, such studies should have some clinical relevance.

Is Visceral Muscle a Niche for ISCs?

Expression of *wingless* (*wg,* a *Drosophila* Wnt) from the visceral muscle (VM) has been reported to regulate ISC proliferation and self-renewal, leading to the proposal that visceral muscle serves as a niche for ISCs (Lin et al., 2008). However, although *Drosophila* Wnt signaling appears to be required for ISC survival (Lin et al., 2008), its role in promoting ISC self-renewal was not confirmed in another independent study (Lee et al., 2009). In addition, ISC proliferation induced by ectopic Wnt signaling is much weaker than that induced by Jak/Stat or EGFR signaling (Jiang et al., 2009; Lee et al., 2009; Lin et al., 2008). Thus, although the role of VM-derived *Wg* in midgut homeostasis and regeneration has not been rigorously tested, the data suggest that other signaling systems play more critical roles.

Pertinent to the function of the visceral muscle, we discovered that the EGFR ligand *vn* was induced in the VM during gut regeneration (Figure 1), and that VM-derived *Vn* was capable of inducing ectopic ISC proliferation (Figure 3A). This suggested that the VM might serve as a part of the ISC niche by providing a mitogenic signal. However, *Pe*-induced compensatory ISC proliferation was not affected when we specifically downregulated *vn* in the VM (Figure 4Q), suggesting that VM-derived *Vn* is probably not by itself an essential EGFR ligand during midgut epithelium regeneration. In fact, we also observed the induction of two other EGFR ligands (*spi* and *Krn*) in midgut epithelial cells during regeneration (Figure 1). Although the concurrent expression of multiple EGFR ligands complicated our efforts to identify the exact role of each ligand, single and double mutant analysis suggested that all three ligands have overlapping function in activating EGFR signaling (Figures 4P and 4Q). Importantly, a significant fraction of the mitogenic EGFR signals probably come from the epithelium itself. Similarly, the *Upd* cytokines are induced primarily in midgut epithelial cells (Buchon et al., 2009a; Jiang et al., 2009). Moreover, the self-renewal and differentiation of *Drosophila* intestinal stem cells are regulated by Notch signaling, which occurs between the two daughter cells produced after ISC division and is not known to directly involve the VM (Bardin et al., 2010; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007).

Therefore we propose that the most important component of the niche for fly intestinal stem cells may be the midgut epithelium itself. In this context it is interesting to note that an epithelial niche has also been proposed for mouse intestinal stem cells (Sato et al., 2009). The murine $Lgr5^+$ ISCs reside at the bottom of the crypts, juxtaposed directly with Paneth cells (Barker et al., 2007). In vitro culture of individual $Lgr5^+$ ISCs has demonstrated that they can form self-organizing organoids in the absence of mesenchymal cells. $Lgr5^+$ ISCs are normally always in contact with Paneth cells, which have been proposed to be a niche for ISCs (Sato et al., 2009). Interestingly, EGF is one of the factors required in the media to support the growth of intestinal organoids (Sato et al., 2009). However, it is not yet clear which cells are the endogenous source for EGFR ligands in the mouse intestine or colon, nor which specific ligands are expressed or functionally important. It is tempting to speculate that Paneth cells, as a critical niche component, might be one of the sources of mitogenic signals, such as EGFs and cytokines, for mammalian intestinal stem cells.

EXPERIMENTAL PROCEDURES

Fly Genetics

See Supplemental Experimental Procedures for fly stocks used in this study.

Upd3-lacZ Reporters

To generate *upd3-lacZ* reporters, four genomic PCR fragments (*upd3.1-4*, see primer sequences in the Supplemental Experimental Procedures) covering the original ~4 kb *upd3* promoter region (Agaisse et al., 2003) were digested with BamHI/KpnI and cloned into the same restriction sites of pH-Pelican vector. Transgenic lines were established through standard P-element-mediated transformation.

RNA In Situ Hybridization in the Adult Midgut

RNA fluorescent in situ hybridization (FISH) in the midgut was performed as described (Raj et al., 2008) with a few modifications. In brief, 40–48 20-mer DNA oligos complementing the coding region of the target genes (*vn*, *krn,* and *rho*) were designed with online software (http://www.singlemoleculefish.com/designer.html). The oligos were synthesized with 3′ amine modification (Biosearch Technologies), then manually pooled and coupled with Alexa-568, carboxylic acid, succinimidyl ester (Invitrogen A-20003). The labeled oligos were purified with HPLC (reverse phase C-18 column) and vacuum dried and resuspended in 100 μl H_2O. For RNA in situ hybridization, the midguts were first dissected and fixed in 8% paraformalhyde overnight at 4°C, then washed with PBS and Triton X-100 (0.1%) for 3 times (15 min each). The samples were further permeablized in 70% ethanol overnight at 4°C. The probes were used at dilution 1:2,000–10,000. The hybridization was then performed according to the online protocol (http://www.singlemoleculefish.com/protocols.html).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and eight figures and can be found with this article online at doi:10.1016/j.stem.2010.11.026.

ACKNOWLEDGMENTS

We thank Celeste Berg, Denise Montell, Gyeong-Hun Baeg, Erika Bach, Jocelyn McDonald, Matthew Freeman, and the VDRC (Austria), NIG (Japan), Bloomington (USA) *Drosophila* Stock Centers for fly stocks; the Moen's lab for confocal imaging; Xiaohang Yang for Pdm-1 antibody; David D. O'Keefe for advice on anti-dpERK staining; and members of the B.A.E. lab for comments. This work was supported by NIH grant R01 GM51186 to B.A.E.

Received: April 20, 2010
Revised: September 20, 2010
Accepted: October 25, 2010
Published online: December 16, 2010

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